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ENDOTHELIAL CELLS AS A SOURCE OF OXYGEN-FREE RADICALS. AN ESR STUDY

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Endothelial cells were subjected to anoxia/reoxygenation in order to simulate some of the free radical mechanisms occurring in ischaemia/reperfusion. With ESR and spin trapping using the spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-dimethyl-1-pyrroline-1-oxide (M_4PO), the results show that upon reoxygenation of endothelial cells, following a period of anoxia, these cells generate superoxide ($O_2^{\cdot-}$). Cytotoxicity of the spin traps was measured by standard trypan blue exclusion methods. Cell injury or death was measured at various times during reoxygenation by lactate dehydrogenase (LDH) release. Experiments using oxypurinol, SOD, CAT and a combination of SOD and CAT show that while oxypurinol partially prevents spin adduct formation, the combination of SOD and CAT is more effective in doing so. These results suggest that the majority of the oxygen radicals produced by endothelial cells are done so exogenously. The results also suggest that endothelial cells are not only a source of oxygen radicals but also a target.

KEY WORDS: Endothelial cells, oxygen radicals, ESR, spin trapping

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INTRODUCTION

In various organs, reperfusion injury following a period of ischemia is, in a substantial proportion, caused by oxygen free radicals.[1,2] Evidence suggests that the oxygen-centered radicals are generated by a similar xanthine oxidase mediated pathway. The fact that different organs demonstrate a similar biochemical mechanism for this injury suggests that the injury may originate from common cellular sources. Possible cellular sources are circulating neutrophils and endothelial cells. Circulating neutrophils are a well-recognized oxygen free radical generating system.[3,4] Endothelial cells, because of their location at the blood-tissue barrier, have been postulated as the initial site of tissue injury during reperfusion.[5] However, it remains unclear if whether initial site refers to the endothelial cells being the target of the reactive free radicals, a source of the reactive free radicals or a combination of both. The role of oxygen metabolites in neutrophil-dependent endothelial cell injury was first suggested by Sacks *et al.*[5] Their experiments showed that neutrophils stimulated with zymosan-activated serum would induce the release of ^{51}Cr from cultured endothelial cells. Furthermore, the ^{51}Cr release was inhibited by superoxide dismutase and catalase suggesting that superoxide and hydrogen peroxide played an important role in the cell damage. Neutrophils

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stimulated by phorbol esters are known to generate superoxide radicals.[3] In addition, Weiss *et al.*[6] has confirmed the ability of reactive oxygen products from activated human neutrophils to damage cultured endothelial cells. With regard to endothelial cells being a possible source of oxygen centered radicals a recent study by Zweier *et al.*[7] has shown, using the technique of spin trapping and the spin trap 5,5 dimethyl-1-pyrroline-N-oxide (DMPO), that oxygen-centered radicals were formed during re-oxygenation of endothelial cell suspensions which had been subjected to anoxia. Although in the study by Zweier *et al.*, scavenging experiments using ethanol implied that $\cdot$OH radicals were formed and experiments with SOD and catalase suggested that the $\cdot$OH radicals originated from superoxide ($O_2^{\cdot-}$), it was concluded, following xanthine oxidase inhibition studies, that the $O_2^{\cdot-}$ was produced endogenously in the endothelial cells.

However, several points were not addressed in this study: (1) How exogenously added SOD suppressed the spin trapping of endogenously generated oxygen radicals. It is well known that exogenously added SOD does not enter cells.[8] (2) How catalase suppresses the spin trapping of oxygen centred radicals. It is known that DMPO reacts with $O_2^{\cdot-}$ and that the DMPO–$O_2^{\cdot-}$ adduct decomposes forming DMPO–OH.[3] Catalase in turn reacts with hydrogen peroxide, a product of the dismutation of $O_2^{\cdot-}$.[3] Although DMPO–OH was observed suggesting the possible production of $O_2^{\cdot-}$ or $\cdot$OH, it is known that this spin adduct may also be formed in the presence of O_2 at room temperature and more rapidly at higher temperatures (37 °C) by processes other than the reaction of $O_2^{\cdot-}$ and $\cdot$OH with DMPO.[9,10] There is also the inherent problem of the DMPO–OH and other nitroxides short-lifetime with cells that has not been addressed.[11] Finally, nothing was mentioned with regard to the origin of additional spin adducts observed in the ESR spectra. It appears that a reasonable amount of the reduction product of DMPO, DMPO–H, was generated in these studies simultaneously with the DMPO–OH.

Therefore, of interest to the present study is to determine whether endothelial cells are actually a source of oxygen centered radicals. In an effort to circumvent the possibility of misassignment of the trapped radicals due to possible artifacts associated with using DMPO, in the present study two spin traps were used: 3,3,5,5-tetramethyl-1-pyrroline-N-oxide (M_4PO) and 5,5 dimethyl-1-pyrroline-N-oxide (DMPO). It is also of interest to verify free radical-induced cell damage, following reoxygenation of anoxic cells, via molecular assays more sensitive than trypan blue exclusion. For this purpose, lactate dehydrogenase (LDH) release from the cells was studied.[12]

METHOD

Endothelial Cell Culture System

Bovine pulmonary arterial endothelial cells (CCL # 207, American Type Culture Collection, Rockville, Md) were cultured in medium 199 supplemented with 18 % fetal bovine serum (GIBCO Grand Island, NY). Primary cultures were maintained on a standard growth medium with penicillin (50 units/ml) and streptomycin (505 g/ml) (Mediatech, Washington, D.C.). Confluent plates were harvested by trypsinizing with 0.25 % trypsin-EDTA (Sigma, St. Louis, MO), centrifuged at 900 rpm (5 minutes). The pellet was washed twice with potassium phosphate buffer (120 mM KCl and

10 mM K_2HPO_4/KH_2PO_4, pH 7.2) at room temperature, then resuspended in the phosphate buffer (6–7 million cells per ml).

The spin trapping studies were performed by using two different spin trapping agents 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-N-oxide (M_4PO) obtained from the Aldrich Co. DMPO as supplied usually contains colored impurities. These were removed using the method described by Buettner and Oberley.[13] Activated charcoal was successively added to a 10 % solution of the nitrone in 10 mM phosphate buffer, pH 7.2, and stirring for 30 min at room temperature. The charcoal was filtered and filtrate was monitored by ESR at high receiver gain (1×10^5) for nitrone impurities. M_4PO was also further purified by charcoal treatment. Anaerobic preparations were achieved by removing the solution above settled cells and adding an identical volume of solution previously purged with nitrogen for 15 to 20 min. Anaerobic conditions were maintained by keeping the cell suspension under a gentle stream of nitrogen gas. Reoxygenation was produced by rapidly removing the nitrogen saturated solution above the cells and replacing it with an equal volume of solution previously purged, for 15 to 20 min, with a gas mixture containing 95 % O_2 and 5 % CO_2. Reoxygenated conditions were maintained by gently exposing the surface of the solutions to an atmosphere consisting of the O_2/CO_2 mixture. The experiments were done in the dark to prevent photolytic degradation of M_4PO or DMPO.

The ESR spectra were recorded on a Bruker IBM ER 100 X-band spectrometer at 100-kHz magnetic field modulation. The ESR spectra were obtained using a quartz flat cell ($60 \times 10 \times 0.25$ mm). The microwave power was maintained at 10 mW to avoid saturation and the modulation amplitude was set at 1 G. Hyperfine coupling constants were measured directly from the spectra using a 10 G marker for calibration. These parameters were also obained by computer simulation, generating theoretical ESR spectra which match the experimentally obtained ESR spectra. The concentration of spin adducts was determined by double integration of the first derivative ESR spectrum. An aqueous solution of 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidine-1-yloxy (Aldrich) was used as the nitroxide standard. This nitroxide has previously been used as a standard for determining unknown free radical concentrations.[14] The accuracy of the double integrations, ± 10 %, was determined using the method described by Hall.[15]

In vitro anoxia/reoxygenation of the cells was used as a model to simulate some of the processes occurring in ischemia/reperfusion. During culture, the cells were normally exposed to atmosphere consisting of 95 % air and 5 % CO_2. Anoxia was produced with an atmosphere of 95 % N_2 and 5 % CO_2, while reoxygenation was produced by restoration of the 95 % air, 5 % CO_2. These atmospheres were produced in a homemade gas chamber approx. 1 l). The chamber was initially purged with each experimental gas for 1 minute at a flow rate of 5 L/min. The experimental time points were taken beginning at the end of each purging. End points were then determined.

Oxypurinol was obtained from Burroughs Wellcome Co. (Research Triangle Park, NC) and was dissolved and used at the desired concentration. Optimal doses of superoxide dismutase (SOD) from bovine erythrocytes, (EC 1.15, 1.1, Boehringer Mannheim Biochemicals, Indianapolis, IN), 5000 Units/mg protein, and catalase (CAT), 5000 Units/mg protein, (Sigma Chemical Co.) were determined individually and together in a dose response fashion. To provide a suitable control for the active SOD, SOD was inactivated according to a modification of the method of Hodgson and Fridovich[16] with overnight dialysis in a glycerine buffer pH 10 with 10 mM H_2O_2.

Similarly, CAT was inactivated by overnight dialysis in 10 mM aminotriazole and 50 mM H_2O_2.

RESULTS

Concentrated suspension of 2×10^7 cells in 0.5 ml were made anoxic by incubation under anaerobic conditions at 37 °C for 45 min. The cells were then reoxygenated by addition of aerobic solutions of DMPO or M_4PO and exposure to atmospheric air. The final concentration of DMPO or M_4PO used was 50 mM. Cells were then immediately transferred to the ESR flat cell and spectra were obtained. When fresh oxygenated buffer containing 50 mM M_4 PO (same identical preparation added to the cells) was monitored, no ESR signal was observed. The spectra of the reoxygenated cells exhibited complex patterns (Figure 1A). The seven line spectrum observed can be explained as two different overlapping ESR spectra which can be simulated as shown in Figure 1C and 1D. Spectrum C consists of a triplet of doublets with hyperfine splittings of $A_N = 15.6$ G and $A_H^{\beta} = 6.5$ G; this spectrum can be attributed to M_4PO–O_2^-. A similar spectrum was obtained in a X/XO solution containing M_4PO or in the photochemical production of O_2^- using riboflavin. The spectral simulation of a triplet with hyperfine splitting constant of $a_N = 18$ G is shown in Figure 1D. The

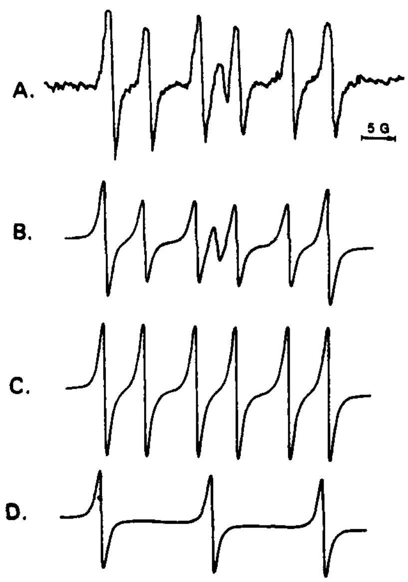

FIGURE 1 ESR spectra of preparations of endothelial cells (1.9×10^7) in the presence of 50 mM M_4PO. A. Reoxygenation following 45 min anoxia at 37 °C. B. Computer simulated spectrum that best fits the experimental spectrum (A). This spectrum is the addition of spectra C and D. C. Simulated spectrum consisting of a triplet of doublets. Hyperfine splittings used in this simulation: $a_N = 15.0$ G and $a_H = 6.5$ G. D. Simulated spectrum of a triplet using $a_N = 18.0$ G as the hyperfine splitting constant. ESR spectra were recorded with a mircowave power of 10 mW and a modulation amplitude of 1 G.

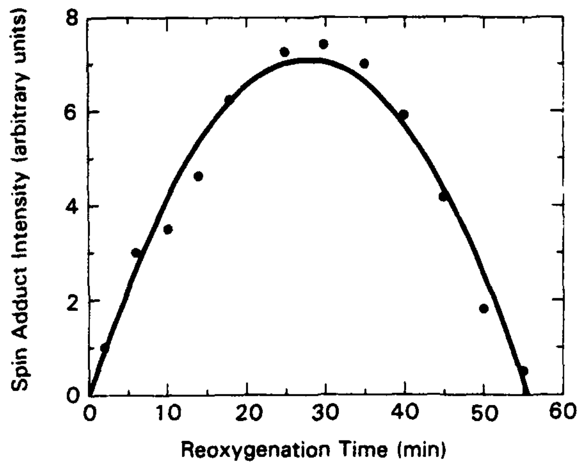

FIGURE 2 Time course of the appearance of M_4PO adducts under identical cell concentration (2×10^7 cells in 0.5 ml) but different reoxygenation time. The concentration of oxygen used in this study was approximately 140 mm/Hg. ESR spectra were recorded every 2 min as described in Figure 1.

simulated spectrum that best fits the experimental spectrum (Figure 1A) is shown in Figure 1B. This spectrum is the addition of the simulated spectra shown in Figure 1C and 1D. These measurements were repeated many times with different preparations of cells and different reoxygenation times. Each time different prominent signals were observed depending on the amount of cells used and the reoxygenation time, with no signal observed in controls. When DMPO (50 mM) was used instead of M_2PO a signal similar to that reported by Zweier[7] was obtained.

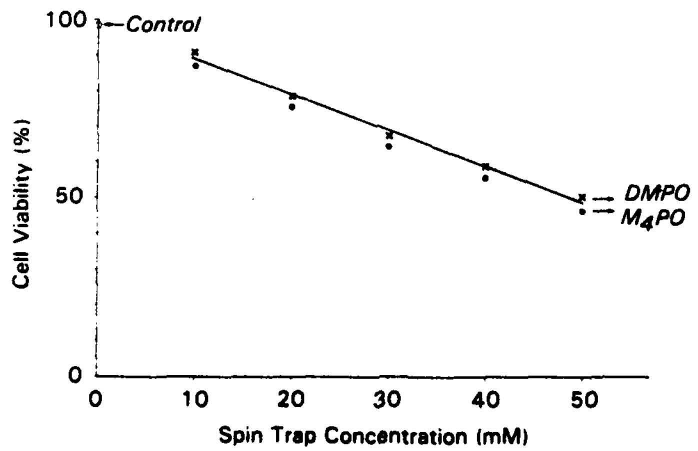

FIGURE 3 Percent of cell viability as a function of M_4PO or DMPO concentration. Spin trap cytotoxicity assay was measured by a standard trypan blue exclusion.

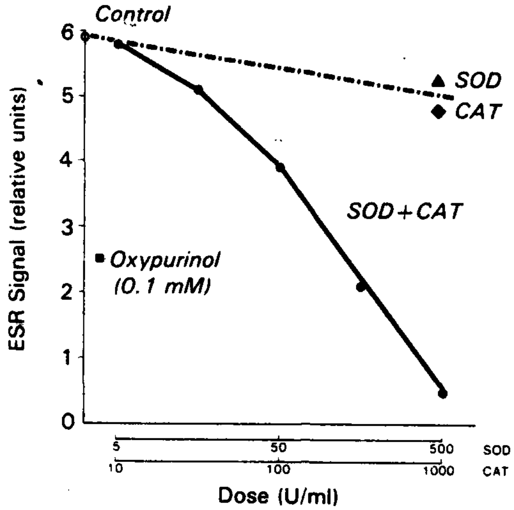

FIGURE 4 Protective effects of varying doses of SOD/CAT, SOD, CAT and oxypurinol in preventing free radical generation monitored by ESR signal intensity. (○) control; (▲) SOD 500 U/ml; (◆) catalase 1000 U/ml; (■) oxypurinol (0.1 mM).

Time course of the appearance of the M_4PO adducts under identical cell concentration (2×10^7 cells/0.5 ml) but different reoxygenation time is shown in Figure 2. The concentration of oxygen used in this study was approximately 140 mg/Hg. These measurements indicated that as early as 1 min after reoxygenation a spin adduct signal was observed. The maximum intensity of the spin adducts observed was at 30 min of reoxygenation. After 50 min of reoxygenation there was no observable signal. A rough quantitation of the maximum observed radical concentration was 1.1 μM was observed in a preparation of 2.1×10^7 cells per 0.5 ml with 50 mM M_4PO.

To determine the fate of the spin adducts observed in Figure 1A, identical experiments were performed to measure the time course of the endothelial free radical generation. The time course of appearance of the M_4PO spin adduct signals in a preparation of 1.9×10^7 endothelial cells in 0.5 ml exposed to 45 min 37 °C anoxia followed by reoxygenation was measured. Immediately after reoxygenation ESR spectra were recorded every two minutes for a period of 1 hr. In time, the seven line spectrum in Figure 1A begins to change generating different hyperfine patterns that could be degradation products of M_4PO or of its spin adducts. Further investigation of this observation is currently being done to determine the nature of these processes.

Spin-trap cytoxicity was measured by a standard trypan blue exclusion test. The endothelial cells were seeded into the wells of a 24-well culture dish at 2.5×10^6 cells per well in 1 ml of culture medium. Each well was treated with a different molar concentration of M_4PO or DMPO ranging from 0–50 mM. The spin trapping agents were allowed to settle onto the endothelial cell monolayer for 30 minutes. Cells were stained with 0.02 % trypan blue in phosphate buffer and cell counts of 100 cells were performed after 3 min with a standard laboratory light microscope. Figure 3 shows the percent of cell viability as a function of M_4PO or DMPO concentration. Cell counts performed in the first 3 min after staining showed that only 48 % of the cells

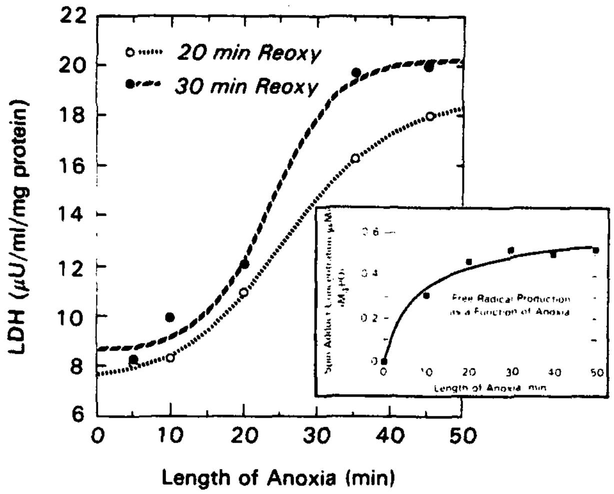

FIGURE 5 Correlation, assessed by the release of LDH, between increasing degree of cell injury with increasing periods of anoxia at 20 min and 30 min reoxygenation. Insert: Radical production monitored by spin adduct concentration as a function of the length of anoxia.

took up trypan blue after being exposed to 50 min of M_4PO for 30 min. 50 % of the cells took up the dye when 50 mM DMPO was introduced for 30 min. Cells not subjected to the spin traps continued to exclude the dye with > 92 % of the cells excluding dye even after 30 min.Figure 4 shows the protective effects of varying doses of SOD and CAT as well as the effect of doses of each agent by itself. The optimal combined doses of SOD and CAT were 500 U/ml and 1000 U/ml, respectively. Neither SOD or CAT alone demonstrated significant inhibition of the ESR signal adducts. Administration of SOD and CAT together substantially and significantly reduced the M_4PO-adduct signals, while neither SOD or CAT alone was effective. The combination of SOD and CAT was equally effective whether administered before the anoxia period or after anoxia just prior to reoxygenation. Inactivated SOD and inactivated CAT had no effect. Oxypurinol (0.1 mM) also provided significant protection but was significantly less effective than SOD and CAT. Since it is known that exogenously added SOD and CAT do not enter the cells, in addition to the observation that oxypurinol only partially surpresses the formation of spin adducts and is less effective than the combination of SOD and CAT, the results suggest that the majority of the oxygen-centered radicals are formed exogenously to the endothelial cells.

The effect of the duration of anoxia on cell injury was examined (Figure 5) at 20 and 30 minutes of reoxygenation. Cell injury increased with an increasing duration of reoxygenation. Also, cell injury increased with an increasing duration of anoxia as measured by the release of LDH. This indicator of cell injury shows a close relationship with the ESR data collected: the maximum intensity of the spin adducts observed was at 30 min of reoxygenation when the cells were made anoxic at 37 °C for 45 min.

Thirty minutes of reoxygenation increased the generation of ESR adducts over that seen with 20 min of reoxygenation. These studies suggest that the generation of M_4PO adducts (free radicals) in reoxygenated endothelial cells parallels gradually increasing

cellular damage as radical production continues. In the presence of combined doses of SOD and CAT 500 U/ml and 1000 U/ml, respectively, cell damage was prevented consistent with an inhibition of free radical generation. Since cell death appears to correlate with radical production, therefore suggesting, that the observed free radical spin adducts may have represented free radicals contributing to cell injury and cell death.

DISCUSSION

These experiments clearly demonstrate that the endothelial cell alone is capable of producing $O_2^{\cdot -}$ upon reoxygenation following anoxia and that this correlates with substantial cellular injury. Interestingly, neither SOD alone nor CAT alone prevented the generation of M_4PO-adducts whereas combination of these two enzymes was very effective. Superoxide dismutase rapidly dismutates superoxide to hydrogen peroxide, which itself is toxic to cells and also has been shown to be an inhibitor of SOD activity.[17] The superoxide radical may also inhibit catalase.[18] Although these properties could explain the need for both scavenging enzymes to be present in order to see inhibition, they are unlikely to occur in the short time frame of the experiments since the H_2O_2 inactivation of SOD is slow and the $O_2^{\cdot -}$ inhibition of catalase is not strong. An exact explanation for the requirement of both enzymes to suppress spin adduct formation is unclear at this time. The fact that SOD and CAT together were as effective when administered just before reoxygenation as when administered before anoxia confirms that the radical production occurs primarily at reoxygenation. Therefore, these studies suggest that the ongoing generation of free radicals in reoxygenated endothelium induce gradually increasing cellular damage as radical production continues.

The ability of oxypurinol to partially inhibit the radical production, as measured by spin adduct formation, suggests that XO is one source of oxygen free radical generation within the endothelial cell at reoxygenation. The fact that oxypurinol was less effective than SOD and CAT combined strongly suggests that the majority of free radicals are generated by other sources that are not inhibited by oxypurinol. However, it must be noted that oxypurinol is a good hydroxyl radical scavenger and in this manner could possibly prevent the observed oxygen radical spin adduct formation. Since exogenously added SOD and CAT do not enter the cells, the results suggest that the production of oxygen-centred radicals that are not inhibited by oxypurinol occurs exogenously to the endothelial cells.

Other studies suggest that the endothelial cell may act as a trigger for postischemic injury. Jarash *et al.*[19] using immunofluorescent techniques, reported that XO was an important constituent of microvascular endothelial cells from a variety of organs.[19] Del Maestro[20] observed direct evidence of microvascular endothelial cell injury in response to the exogenous generation of the superoxide with X/XO. Therefore, it appears that the initial injury occurs within or near the intravascular space. For instance, SOD is effective in preventing postischemic reperfusion injury in the cold-preserved, allotransplanted kidney when administered intravascularly at the time of reperfusion. On the other hand, when the kidney is stored in a high concentration of SOD and SOD is not added to the vascular stream at reperfusion, no protection is found.[21]

The technique of spin trapping involves producing the unstable free radical of

interest and allowing it to react with a diamagnetic compound (a nitroso or nitrone compound) to form a more stable free radical which can be observed by electron spin resonance (ESR). The observation of a stable free radical, however, is no guarantee that the radical of interest has been trapped. Spectral artifacts can arise due to nitroxide impurities or nucleophilic addition to nitrone compounds followed by oxidation of the nitroxide. The triplet spectrum observed in these studies reflects another way in which spin trapping artifacts may arise, that of direct reduction of a nitrone spin trap to a nitroxide free radical. Though nitrone spin traps have been used extensively, little attention has been given to the possibility of this reduction, though reduction of the spin adduct has been proposed as a decay process.[22]

The reduction of the spin trap itself or of the spin adduct is particularly important in biological systems because of the presence of endogenous reducing agents such as ascorbate. Therefore, the data presented here must be viewed critically to ensure that conclusions are not drawn on the basis of artifacts. The fact that the triplet signal of the M_4PO was generated in the presence of the endothelial cells only when they were subjected to anoxia followed by reoxygenation, implies that some biological reducing agents are generated during anoxia/reoxygenation, an important part of the conditions observed in ischemic and reperfused tissue.

In the present study we showed that bovine pulmonary artery endothelial cells could be a source of toxic oxygen products, including O_2, OH and other species undefined. A likely explanation, which is consistent with the published data, is that superoxide generation from activated XO within the endothelial cells trigger the biochemical mechanism at reperfusion. The fact that superoxide generation from anoxia/reperfusion conditions can be observed by spin trapping (M_4PO-$O_2^{\cdot-}$ adduct) establishes its independence as a source.

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